STUDIES IN THE REGULATION OF BLOOD-SUGAR CONCENTRATION IN CRUSTACEANS. I. NORMAL VALUES AND EXPERIMENTAL HYPERGLYCEMIA IN LIBINIA EMARGINATA

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Introduction

Studies of blood-sugar concentrations of invertebrates were, until relatively recently, confined for the most part to defining the range of glycemic values in different species. Stimulated by investigations of the effects of insulin on blood-sugar concentrations in mammals, these early studies among invertebrates demonstrated that considerable variations existed in the amount of glucose in the blood, even within a single species. The reviews of Beutler (1939) and of Benazzi-Lentati (1941) summarize many of these observations.

The wide range of glycemic concentrations in crustaceans reported by different investigators, who at times studied the same species, was soon recognized to be due, in part, to the different analytical methods employed and, perhaps even to a greater extent, to the varied physiological states of the animals at the time blood samples were taken for analysis. This latter possibility led to observations on animals maintained under more critical laboratory conditions and to studies of factors that influenced the amount of sugar in the blood.

Thus, where Hemmingsen (1924a) had found an increase in blood sugar in Astacus after feeding, Kisch (1929) reported a decrease in *Carcinus maenas* during starvation. These results were confirmed both by Stott (1932) and by Florkin (1936) for the same species of Carcinus. On the other hand, Roche and Dumazert (1935) found that the blood glucose of *Cancer pagurus* starved for one month did not differ significantly in concentration from that of freshly captured individuals. Asphyxiation was reported by Stott (1932) to cause marked hyperglycemia in *C. pagurus, Portunus puber,* and *Carcinus maenas;* this observation was confirmed by Roche and Dumazert (1935) on *Cancer pagurus.* Stott (1932) also observed a high concentration of glucose in the blood of newly-molted crustaceans (within a few hours of ecdysis) compared with animals before the molt.

Hemmingsen's studies (1924b) on the crayfish Astacus led him to believe a regulatory mechanism was present for maintaining a constant level of blood sugar. The basis for this view was his observation that samples of concentrated glucose solution when injected into Astacus disappeared from the blood stream too rapidly to have been oxidized to CO_2 during the experimental period, and yet no glucose was excreted in detectable amount into the water in which the injected animals

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were maintained. These results Hemmingsen regarded as evidence for a hypoglycemic regulatory mechanism.

In addition, a number of pharmacological substances had been reported as being effective in inducing hyperglycemia in crustaceans. The controversy in this branch of the general problem lay not so much in the interpretation of such hyperglycemias when they occurred, but whether these substances really induced hyperglycemia. Thus, Medvédéva (1936) reported that injection of adrenalin in Potamobius (Astacus) caused hyperglycemia, but that injection of insulin was without any definite effect. Roche and Dumazert (1935), on the other hand, reported that neither adrenalin nor insulin had any appreciable effect on the blood-sugar concentration of *Cancer pagurus*. Kalmus and Waldes (1936) stated that not only adrenalin and insulin, but also such non-specific substances as hydroquinone and sodium chloride solution, effected marked hyperglycemias when injected into crayfish. Florkin and Duchateau (1939), using more carefully controlled procedures, reported that insulin had no effect while adrenalin produced a hyperglycemia in the crayfish, thus confirming the observations of Medvédéva.

These scattered observations, controversial though they may have been, indicated the possible existence of a hyperglycemic mechanism, just as Hemmingsen's studies had indicated the possibility of a hypoglycemic mechanism. The first indication of a definite anatomical structure which might be involved in regulating sugar metabolism in crustaceans was made by Abramowitz, Hisaw and Papandrea (1944). These authors found that injection into *Callinectes sapidus* of aqueous extracts of crustacean eyestalks increased the concentration of blood sugar within an hour. More specific localization of the source of this diabetogenic factor was demonstrated by the preparation and injection of extracts prepared from the sinus glands of Hanström that had been removed from eyestalks. The injection of such extracts resulted in a marked hyperglycemia amounting to nearly four times the normal basal concentration of blood glucose. When extracts, prepared from the remainder of the eyestalks from which the sinus glands of Hanström had been previously removed, were injected, they were practically without hyperglycemic effect. These investigators' complementary experiments, which consisted of removing the sinus glands by ablation of both eyestalks, to determine whether hypoglycemia would ensue, gave negative results; in fact, over a period of seven days after eyestalk removal, there was an anomalous, slight increase in concentration of blood sugar.

Our own studies were undertaken to define in greater detail the nature of such glycemic changes under experimental conditions and to investigate the physiology of the regulatory processes. This is the first report in detail of our investigations, some of which have appeared in abstract form (Kleinholz, 1948; Kleinholz and Little, 1948; Kleinholz and Havel, 1948).

Materials and Methods

The animals used in this study were the marine spider crab, *Libinia emarginata*. A large stock of animals was maintained by the laboratory collectors in a live-car. Other than the occasional placing of a freshly killed fish into the live-car, no regular feeding of these stock animals was undertaken. When groups of animals were removed for use in the laboratory, they were starved for three days before

blood samples were taken, to insure a basal level of blood-sugar concentration, and were not otherwise fed except where indicated. Such experimental animals were marked for identification by painting serial numbers with lacquer on the dorsal surfaces of the carapace of each; the crabs were then placed in individual containers, similarly numbered, through which a stream of sea water circulated. It was hoped that the hyperglycemic effect of crowding reported by Abramowitz et al (1944) would be reduced or obviated by such isolation. Only male individuals were used in this study.

Tuberculin hypodermic syringes of 1 ml. capacity, graduated in hundredths of a milliliter, were used for taking blood samples. Each syringe was calibrated to deliver 0.5 ml. by weighing the volume of water delivered between the 0.60 ml. and 0.10 ml. marks on the barrel of the syringe. Blood for analysis was taken from the sinuses of the walking legs, the arthroidal membrane between the base of the leg and the body being first wiped dry with filter paper or absorbent cotton. The syringe was filled slightly beyond the 0.60 ml. mark and, after withdrawing the needle from the sinus, was emptied to this mark, the excess droplet of blood being removed by touching the tip of the needle to filter paper. A 0.5 ml. sample of blood could thus be delivered into tubes containing the deproteinizing mixture. To avoid injury to the arthroidal membrane that would ensue from the repeated bleeding of the same individual, samples were taken from different legs on both sides of the animal. In all of the experiments reported here, blood samples from control and from experimental animals were taken in the daytime, in most cases in the forenoon. The possibility of a diurnal variation in concentration of blood sugar was thus avoided.

It was found most convenient to work with groups of six Libinia at a time. In most instances blood samples were taken from the individuals of a group before a particular treatment, and then again after the experimental treatment, each crab thus serving as its own control. The control blood samples and the experimental blood samples were carried through the analytical procedure simultaneously and received comparable handling. The method for determining the amount of blood glucose was that described by Miller and Van Slyke (1936). It is reported that this method, when used with mammalian blood, gives "true" blood-sugar values, which do not include non-fermentable reducing substances. We have found a significant amount of non-fermentable reducing substance present in Libinia blood, so that in our hands the method must be considered as expressing *total reducing substances* as glucose equivalents. The procedure was essentially as described by Miller and Van Slyke. The dilute ceric sulfate for the titration was prepared fresh daily from the stock solution.

Two blanks, consisting of the reagents used in the glucose determination, were used with each set of blood samples. These blanks required about 0.15–0.25 ml. of the dilute ceric sulfate to reach the same end-point obtained in the titration of the blood samples. This wide range was due to the preparation of a second lot of stock reagent solutions during the course of the work. For the first set of stock solutions the blanks varied from 0.18–0.25 ml., the average for 38 blanks being 0.22 ml.; with the second set of stock solutions the blanks varied from 0.13–0.18 ml., the average for 20 blanks being 0.15 ml. The average for the total 58 blanks was 0.195 ml. of the dilute ceric sulfate.

The accuracy of the Miller and Van Slyke method in our hands was tested by determining the amount of glucose in prepared solutions of known concentration. These concentrations ranged from 16–200 mg. of glucose per 100 ml. of solution. The average percentage of error for 13 such determinations was ± 3.9 per cent.

Where Libinia without eyestalks were used to determine the effect of absence of the sinus glands on the blood-sugar level, bilateral eyestalk ablation was done with the aid of fine dissecting scissors. Bleeding from the cut surface was very slight and ceased upon the formation of a blood clot in the orbit.

The distinction between total reducing substances and non-fermentable reducing substances in the blood was made by fermenting one of two blood samples with a 10 per cent suspension of Fleischmann's yeast. The yeast was prepared by suspension in distilled water, centrifuging, and pouring off the supernatant. After three such washings, the final 10 per cent suspension was kept in the refrigerator until used. In the fermentation, 3.5 ml. of the yeast suspension and the 0.5 ml. blood sample were mixed and allowed to remain at 22° C. for one hour, after which the mixture was centrifuged and the supernatant poured off into a second tube containing acid cadmium sulfate. The yeast and blood residue was similarly washed and centrifuged three times with 1 ml. portions of distilled water, the supernatants each time being added to the first one. A second 0.5 ml. blood sample taken from the same animal had been prepared for the routine analysis. Both blood samples were carried through the analytical procedure simultaneously, using adequate blanks (washings of a 3.5 ml. aliquot of yeast suspension) for the fermented samples.

The reliability of this method of fermenting glucose in blood was tested with samples from six Libinia. To a 0.5 ml. portion of blood from each animal was added 0.5 ml. of solution containing 5.06 mg. of glucose (thus equivalent to adding 1012 mg. per cent of glucose to the blood sample) and 3.5 ml. of the 10 per cent yeast suspension. The blood samples were fermented and treated as described above. The glucose-equivalent in reducing substances present in these samples ranged from 6.3–11.1 mg. per cent, with an average of 8.9 mg. per cent, showing practically complete fermentation of the added glucose.

Observations

A. Normal and eyestalkless animals

The studies of Abramowitz, Hisaw and Papandrea (1944) pointed to the sinus glands as being mediators in the hyperglycemic response following injection of prepared extracts. Their attempts to observe whether removal of this gland (by ablation of both eyestalks) resulted in hypoglycemia yielded paradoxical results, a gradual hyperglycemia being observed in such animals over a period of seven days.

Our own observations made over a longer period in a comparable series of experimental animals, do not confirm the latter results of these investigators. Of eighteen animals brought into the laboratory at the same time, the eyestalks of twelve were ablated, while the remaining six served as normal control animals. The animals of the control group were isolated in individual containers; six of the operated Libinia were designated as Group A and were similarly isolated, while the remaining six operated animals, constituting Group B, were placed in a com-

mon tank. These animals were not fed during the time the experiment was in progress. Beginning on the morning of the third day after eyestalk removal, blood samples were taken from the individuals of Group A and of the control group; on the following morning, the fourth day after ES removal, samples were taken for analysis from individuals of Group B. By alternating in this fashion and taking blood samples every third day, the observations were extended over a period of twenty-six days. The results which are shown in Table I are the averages and the standard deviations for the six animals constituting each group.

TABLE I

Comparison of the blood-sugar concentrations in two groups of Libinia without eyestalks, with that of a normal control group. The figures are the averages for the 6 crabs of each group and their standard deviations.

Days after eyestalk removal	Blood-sugar concentration in mg.-per cent		
	Group A	Group B	Control
3	10.1 ± 2.5	—	6.9 ± 1.2
4	—	11.1 ± 2.1	—
6	12.0 ± 3.1	—	9.9 ± 1.5
7	—	9.0 ± 2.5	—
10	10.2 ± 3.4	—	7.4 ± 1.3
11	—	9.3 ± 3.6	—
17	10.2 ± 4.3	—	7.3 ± 1.8
18	—	9.6 ± 3.7	—
24	10.1 ± 3.2	—	8.7 ± 1.7
26	—	7.9 ± 2.6	—

As might be expected, there were variations in the concentration of blood sugar not only among the individuals of a group, but also in the same individual at the different intervals when blood was taken for analysis. These variations are probably due to a combination of actual fluctuations in glucose concentration and of slight artifacts in the analytical procedure. The relatively low average of the blood-sugar concentration in the control group on the third day, compared with later averages, is probably to be explained on this basis, for the average glycemic values determined for three different normal groups, which had been starved three, six, and ten days, were, respectively, 7.9, 8.3 and 9.1 mg. per cent. We conclude from the data of Table I that removal of the sinus glands has no marked effect on the basal level of the blood-sugar concentration. The hyperglycemia reported by Abramowitz et al. after eyestalk removal in Callinectes could not be confirmed with Libinia.

B. Total reducing substances and true blood sugar

It has been known that the blood of mammals contains, in addition to glucose, other reducing substances which, in the analytical methods currently employed, may contribute significantly to the total value obtained as "apparent" glucose. For an accurate measure of the amount of glucose in a blood sample the supple-

mentary use of a yeast fermentation method along with the conventional determination permits the distinction to be made between total reducing substances or apparent blood glucose, and non-fermentable reducing substances; the difference between the two such determinations is then considered to represent the fermentable glucose.

The application of methods devised for the analysis of mammalian blood to that of invertebrates would require similar supplemental yeast fermentation methods, since little is known about the presence or the nature of non-fermentable reducing substances in blood of the latter group. Such yeast fermentation analyses of the blood of Libinia, using the procedure described under "Methods," were conducted in parallel with samples taken at the same time for the determination of total reducing substances. The results for experimental and control animals are arranged in Table II.

TABLE II

True blood-sugar concentrations in groups of eyestalkless and of normal Libinia. Each group consisted of 6 animals. TRS, *total reducing substances;* NFRS, *non-fermentable reducing substances;* TBS, *true blood sugar. Figures are averages for the animals of a group and the standard deviations from the mean.*

Animals and condition	Concentration in mg. per 100 ml. blood		
	TRS	NFRS	TBS
Group A, starved 24 days	10.1 ± 3.2	7.4 ± 0.8	2.7
Group B, starved 26 days	7.9 ± 2.6	5.0 ± 1.3	2.9
Controls, starved 24 days	8.7 ± 1.7	6.8 ± 1.7	1.9
Normal, starved 6 days	8.3 ± 1.3	6.3 ± 0.7	2.0

The data shown in this table represent the averages for four groups of Libinia, each group consisting of six animals. Three of these groups consisted of individuals whose blood analyses for total reducing substances had been made at intervals over nearly four weeks, as shown in Table I. The animals of Group A, Group B, and the control group are described in the text above and in the preceding table. On the twenty-fourth and twenty-sixth days when final analyses were being made on these three groups, additional samples were taken at the same time for determination of the non-fermentable reducing substances after the blood sample had been mixed with yeast suspension and fermented. The fourth group of Table II consisted of normal animals which had been starved six days, in comparison with the twenty-four days of starvation undergone by the control group to the eyestalkless condition. The figures which are given for concentration of true blood sugar are averages for the six individuals of a group, representing the differences between the average concentrations of total reducing substances present in one set of samples and the average amounts of non-fermentable reducing substances found in similar samples after they had been fermented by the yeast suspension.

In all groups the amount of true blood sugar is quite low. There appears to be no difference in glycemic level between normal animals starved for a short period (6 days) and those starved for an appreciably longer time (24 days). At

first glance the slightly higher level of true blood sugar in the groups of eyestalkless individuals as compared with that in the normal control animals might seem to indicate an alteration in glucose metabolism as a result of eyestalk removal, but in view of the small number of animals involved in the experimental groups and the comparatively high standard deviations of the averages for each group, it is doubted that these differences from the controls can be regarded as significant.

C. Hyperglycemia as a result of injection of eyestalk extract

The hyperglycemic effects obtained by Abramowitz, Hisaw and Papandrea (1944) in Callinectes upon injection of eyestalk extracts showed a rough agreement between dosage and the increment of the resulting hyperglycemia. But since their determinations were in terms of total reducing substances, with no distinction being made between fermentable and non-fermentable components, closer examination was made of these components of the total reducing substances at the same time that we tried to confirm their observations.

Blood samples from a group of six Libinia from which both eyestalks had been ablated were analyzed on the first and fifteenth days after eyestalk removal. At these times the average concentrations of total reducing substances were respectively 7.1 ± 1.5 mg. per cent and 6.3 ± 1.6 mg. per cent, confirming previous observations made in Table I that no significant change follows in the glycemic level of animals from which both eyestalks have been removed. On the morning of the seventeenth day after eyestalk ablation, each of the crabs was injected with 0.1 ml. of extract prepared from the eyestalks of Libinia, so as to receive the equivalent of one eyestalk. One hour after the injection, blood samples were taken from each animal for determination of both the total reducing substances and the non-fermentable reducing substances. Five of the six injected Libinia showed striking increments in total reducing substance in the blood, the concentrations after injection being from twice to nearly five times those obtained before injection; in the sixth animal the amount of total reducing substance after injection was about 60 per cent greater than before the injection. The average value for total reducing substance for all six animals was 17.3 ± 6.4 mg. per cent. The average for non-fermentable reducing substances of the post-injection samples after yeast treatment was 4.5 ± 0.4 mg. per cent; average fermentable blood sugar after injection was therefore 12.8 mg. per cent. Yeast fermentations were not made on blood samples taken on the first and fifteenth days after eyestalk removal, but if the average value for true blood sugar is assumed to be comparable to those shown in Table II, then the average increase in fermentable blood sugar after injection of eyestalk extract is well over 400 per cent. We are thus able to confirm the observation of Abramowitz et al. that injection of crustacean eyestalk extract induces a marked hyperglycemia in crustaceans, and to show, furthermore, that this increase is apparently a fermentable sugar.

D. Hyperglycemia as a result of asphyxia

Stott (1932) had reported a large increase in blood sugar of crustaceans which had been kept for ten hours in containers of sea water that had been tightly covered. This change he attributed to asphyxia due to the decrease in oxygen content of the

water during the period of the experiment, because when an adequate air supply was again made available to the animals by removing the cover, the blood-sugar concentration returned to the normal level. Roche and Dumazert (1935) confirmed this observation by reporting that removing animals from sea water and keeping them in air for 30–60 minutes resulted in a marked hyperglycemia. Both studies reported this hyperglycemia as a direct observation, with no attempt to investigate in further detail the mechanism of this response.

Similar results were obtained by us with Libinia, and a possible mechanism for what we shall call the hyperglycemia of asphyxia was indicated by further study. In these experiments, groups of six normal and six eyestalkless animals were employed. To obtain partial asphyxia during which the animals could be kept under observation, the method of Roche and Dumazert was used: removing the animals from sea water and keeping them in air for 60 minutes, and then removing a blood sample from each for analysis and for comparison with samples before asphyxia. The results of these experiments are shown in Table III.

TABLE III

Effect of asphyxia on the blood-sugar concentration of normal animals and animals without sinus gland

Animal group	Eyestalk condition	Days starved	Concentration in mg.-per cent	
			Before asphyxia	After asphyxia
Group B Nos. 13–18	ES off 31 days	31	(7.9 ± 2.6)*	7.0 ± 2.8
Controls Nos. 19–24	Normal	31	(8.7 ± 1.7)*	16.1 ± 8.3
Nos. 31–36	ES off 1 day	18	7.1 ± 1.5	6.0 ± 1.1
Nos. 25–30	Normal	19	7.3 ± 1.0	22.0 ± 12.2

* See Table I.

The first two groups of crabs tested consisted of eyestalkless individuals which had been under observation for several weeks (the animals constituting Group B of Table I), and a similar number of normal Libinia which had been their controls. Both groups were removed from sea water and placed in individual finger-bowls in air. After one hour of such exposure, blood samples were taken for analysis (at this time the animals were limp and showed a marked loss of muscular tone; following their return to sea water recovery was rapid). No blood samples were taken in this experiment directly before the asphyxiating experience, the glycemic values which had been determined at regular intervals for the preceding twenty-six days being considered sufficient to serve as a standard. As can be seen from Table III, the effect of this asphyxia was different in the two groups, the crabs without eyestalks showing no appreciable change in their average concentration of blood sugar, while the group of normal animals showed a marked increase in the glycemic average for the group.

The experiment was then repeated with two additional groups of similar animals. This time a blood sample was removed before subjecting the animals to asphyxia, and the second sample was taken immediately after the 60 minutes of asphyxia. The results were similar to those obtained previously; the normal animals (with eyestalks) showed a marked hyperglycemia, the average concentration being three times the pre-asphyxia level, while the animals without eyestalks showed no significant change from the glycemic level before asphyxia. These results show that the observed hyperglycemia is dependent upon the intact eyestalk and indicate the possibility that the response may be mediated by the sinus gland. The results of more exact studies, in which sinus glands were removed from otherwise intact eyestalks, to define the mechanism of the hyperglycemic response, will be reported later.

E. Alimentary hyperglycemia

A number of investigators have reported the effects of feeding and inanition upon blood-sugar levels in crustaceans. The studies of Henmingsen (1924a) and Stott (1932) showed that feeding resulted in a rise in blood-sugar concentration. Stott had found that in a group of starved *Carcinus maenas,* the glycemic level ranged between 5–8 mg. per cent. When such animals were fed mussels, the blood sugar rose to values of 20 mg. per cent or more over a period of several hours; about fourteen hours after such feeding, the level of blood sugar returned to a concentration of approximately 5 mg. per cent.

In view of the part played by the eyestalk and sinus gland in mediating the hyperglycemia resulting from asphyxia, as described in the preceding section, it was thought advisable to determine whether alimentary hyperglycemia was similarly regulated. Seven Libinia from which both eyestalks had been removed three days previously, and which had been starved for three days, were isolated in individual containers. The average glycemic value immediately before feeding was 9.1 ± 2.8 mg. per cent. Each animal was then supplied with 5–10 grams of the visceral mass of *Venus mercenaria,* which was devoured within fifteen minutes. Blood samples taken three hours after this feeding showed a marked rise in sugar content in each of the seven animals, the average for the group after feeding being 18.3 ± 4.7 mg. per cent. The results therefore indicate that alimentary hyperglycemia is not mediated by the sinus glands in the eyestalks.

Summary

1. Removal of the sinus glands by eyestalk ablation in unfed *Libinia emarginata* has no significant effect on the blood-sugar concentration when compared with similarly unfed controls.

2. Values for true blood sugar, as distinguished from total reducing substances, were determined after yeast fermentation of blood samples. In starved animals the concentration of total reducing substances is between 8–9 mg. per cent; that of non-fermentable reducing substances, 6–7 mg. per cent; that for true blood sugar is therefore about 2 mg. per cent.

3. Injection of eyestalk extract increases the concentration of total reducing substances in the blood. This increase is in the fermentable component, amounting

to over 400 per cent of that in the uninjected animal, and therefore probably represents a true hyperglycemia.

4. Asphyxia also causes hyperglycemia, the total reducing substances in blood samples being two to three times the concentration preceding asphyxia.

5. Removal of the sinus gland by eyestalk ablation prevents the appearance of the hyperglycemia of asphyxia. The sinus gland may be a mediator in certain hyperglycemic responses of crustaceans, but does not seem to be concerned in alimentary hyperglycemia.

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